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Research Article

Mapping the northernmost transnational non-native population of *Xenopus laevis* using pooled eDNA sampling

Teun Everts[1,2*], Loïc van Doorn[3*], Tim Adriaens[4], Sabrina Neyrinck[1], Kevin Kerckhofs[1], Jeroen Speybroeck[3], Rein Brys[1]

1 *Research Institute for Nature and Forest (INBO), Genetic Diversity, Geraardsbergen, Belgium*
2 *KU Leuven, Department of Biology, Plant Conservation and Population Biology, Heverlee, Belgium*
3 *Research Institute for Nature and Forest (INBO), Monitoring and Restoration of Aquatic Fauna, Linkebeek, Belgium*
4 *Research Institute for Nature and Forest (INBO), Wildlife Management and Invasive Species, Brussels, Belgium*

Corresponding author: Teun Everts (teun.everts@inbo.be)

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Abstract

Early-detection-rapid response (EDRR) programs are invaluable for managing incipient invasions, but require monitoring strategies that maximize search coverage and minimize costs. While environmental DNA (eDNA)-based monitoring techniques are now widely used for monitoring aquatic invasions, the large monitoring efforts in EDRR programs demand further improvements in sampling strategies. This need is exemplified by the recently discovered African clawed frog *Xenopus laevis* (Daudin, 1802) invasion along the French-Belgian border region – its northernmost introduced population – where it was first detected in France in 2018 near the Belgian border. In response to the risk of its permanent establishment and cross-boundary spread, we employed quantitative eDNA barcoding analyses in the context of an EDRR program. We (i) developed and validated a novel cost-efficient sampling strategy that involves pooling water from multiple sites allowing for broader area coverage, (ii) delineated the spatial extent of the invasion, and (iii) assessed whether lotic systems serve as cross-boundary dispersal corridors. In 2020, 2022, and 2023, we determined the presence and eDNA concentrations of *X. laevis* from a total of 426 sites by pooling water from 366 sites in 83 samples, and sampling 59 additional sites individually. We found that this pooled approach can accurately determine *X laevis* presence and approximate its average eDNA concentration across pooled sites. We detected *X. laevis* in 26 samples, revealing an interconnected population spanning an area of 103 km^2, contradicting prior assumptions of an early invasion stage. We provide evidence that the river Lys and the Douvebeek facilitate cross-boundary movement. The pooled sampling technique presented here is a cost-effective method for providing timely, actionable data to inform management decisions; however, further validation is needed to confirm its reliability and broader applicability. The *X. laevis* invasion along the French-Belgian border underscores the complexities of managing biological invasions across national and administrative boundaries, providing valuable insights for other transboundary invasions.

Key words: African clawed frog, biological invasions, dispersal corridor, droplet digital PCR (ddPCR), early-detection-rapid-response (EDRR), environmental DNA (eDNA), non-native invasive species, rapidly expanding distribution

* Contributed equally as the first author.

Introduction

Freshwater ecosystems harbour ten percent of all species and one-third of all vertebrate species while covering less than one percent of the Earth's surface (Dudgeon et al. 2006). However, freshwater biodiversity is declining at an alarming rate (Sayer et al. 2025), with the ongoing establishment and spread of non-native invasive species (NIS) identified as a significant contributor. These biological invasions can result in species extinctions, biotic homogenization, and disruptions to ecosystem functioning (Katsanevakis et al. 2014; Blackburn et al. 2019; van Rees et al. 2021; Andrés et al. 2023). Despite ongoing management efforts, the influx of NIS is expected to continue (Seebens et al. 2017). To counter newly incoming NIS, establishing early-detection-rapid-response (EDRR) systems is essential (Hulme 2006; Vander Zanden and Olden 2008). To be effective, EDRR systems must employ cost-efficient monitoring methods capable of detecting species at low densities and having the potential to operate effectively across extensive spatial scales (Myers et al. 2000; Mehta et al. 2007; Greenhalgh et al. 2022).

In recent years, a growing body of evidence has demonstrated the effectiveness of environmental DNA (eDNA)-based analyses for the early detection of aquatic NIS, underscoring their value in EDRR systems (Trebitz et al. 2017; Everts et al. 2023). Typically stemming from skin cells, mucus, gametes, and other biological materials, eDNA represents the entirety of DNA directly extractable from environmental samples, including water, soil, and air, eliminating the necessity to observe or capture the organism itself (Ficetola et al. 2008). eDNA-based detection methods are generally more cost-efficient and sensitive compared to conventional monitoring methods – such as netting, acoustic monitoring, or visual surveys – particularly when surveying inaccessible habitats or targeting cryptic species (Fediajevaite et al. 2021; Keck et al. 2022; Moss et al. 2022). Beyond species detection, eDNA-based analyses can, in certain circumstances, also provide approximate estimates of species densities (Yates et al. 2019; Rourke et al. 2021; Carvalho et al. 2022; Everts et al. 2022). However, the accuracy of eDNA-based analyses can vary significantly depending on factors such as the specific ecosystem and target species (Fediajevaite et al. 2021; Keck et al. 2022). Interpreting eDNA data in open systems, such as marine or riverine environments, can be more complex than in closed environments such as ponds or small lakes due to intricate effects of admixture, diffusion, and mixing on the distribution of eDNA particles (Rourke et al. 2021; Hinz et al. 2022). Even within a given system, the performance of eDNA-based monitoring can be affected by species-specific factors, including eDNA shedding rate, species density, phenology, and mobility (Adams et al. 2019; Everts et al. 2021; Thalinger et al. 2021). Overall, eDNA-based methods are expected to provide the greatest value in detecting early invasion stages of fish and amphibian species in ponds or small streams (Secondi et al. 2016; Fediajevaite et al. 2021; Everts et al. 2022; Keck et al. 2022).

The African clawed frog, *Xenopus laevis* (Daudin, 1802), serves as a pertinent example of a NIS for which an eDNA-based EDRR system may prove highly effective (Measey et al. 2012; Secondi et al. 2016; Vimercati et al. 2019). Native to Sub-Saharan Africa, *X. laevis* has been used as a model organism in biological research and pregnancy testing for decades (Gurdon and Hopwood 2000; Wallingford et al. 2010). It has also become a lucrative commodity in the pet trade (Measey 2016), leading to its global exportation in the 20th century and to the successful establishment of populations in various regions across North-America,

South-America, Asia, and Europe. Given its aquatic lifestyle, effective camouflage, and underwater mating calls (Tinsley and Kobel 1996), introductions of *X. laevis* often remain unnoticed for extended periods (Measey et al. 2012). The species primarily breeds in ponds and disperses via streams and rivers (Moreira et al. 2017), with periodic overland movement that typically occurs during wet periods and heavy rainfall (Measey et al. 2012; Measey 2016). *Xenopus laevis* has a largely aquatic ecology, can adapt to a wide range of environments, is highly fertile, and can disperse up to 5.4 kilometres per year (Pagano et al. 2024). It can exert a profound impact on native aquatic fauna due to predation, competition, and disease transmission (Weldon et al. 2004; Lillo et al. 2011; Courant et al. 2018). The cryptic and invasive nature of *X. laevis* makes it a highly suitable target for eDNA-based monitoring systems, which offer a sensitive and cost-effective approach for generating critical information to support the management of biological invasions (Secondi et al. 2016; Brys et al. 2021; Everts et al. 2022, 2023, 2024).

In Europe, established *X. laevis* populations can be found in Portugal, Italy, and France (Measey et al. 2012). In France, *X. laevis* was first recorded in the western department Deux-Sèvres in the 1980s (Fouquet and Measey 2006). Since then, the species has spread at an estimated rate of 1.2 km per year, reaching an invaded area of 8,400 km^2 by 2024 (Pagano et al. 2024). Further north, a new population was discovered in 2018 in the Armentières canton (Nord department, France), just a few kilometres from the Belgian border (van Doorn et al. 2022). By 2021, the presence of *X. laevis* had been confirmed in two adjacent administrative regions of Belgium – Flanders and Wallonia (Adriaens et al. 2023; Pauwels et al. 2023). However, the spatial extent of this new invasion in Belgium, as well as the pathways facilitating cross-boundary expansion, remained largely unknown. To address this knowledge gap, we sampled 426 water bodies in the French-Belgian border region between 2020 and 2023 for quantitative eDNA-based barcoding analyses to determine the presence and associated eDNA concentrations of *X. laevis* throughout the area. We developed and preliminarily tested the effectiveness of a novel sampling strategy that pools water from multiple sites (Fig. 1), enabling extensive area coverage in a short timeframe with limited resources, and discuss its strengths and limitations. We mapped the current distribution of *X. laevis* in the border region as a part of an EDRR program, and assessed whether two waterways – the river Lys and its tributary, the Douvebeek – serve as potential dispersal corridors (Pauwels et al. 2023). Given that *X. laevis* is currently regarded as being in an early invasion stage where complete eradication might still be feasible (Reniers et al. 2023), the insights gained from this study could support effective transboundary management strategies. We further address the challenges posed by the expansion of NIS across national and administrative borders and propose potential solutions to mitigate these issues.

Material and methods

Study area

The French-Belgian border region is characterized by a temperate oceanic climate with warm summers, but is substantially colder compared to other European areas containing established non-native *X. laevis* populations. Here, vast agricultural fields are interspersed with small towns (Fig. 2), similar to other regions in Europe that are invaded by *X. laevis* (Measey et al. 2012). The study area contains numer-

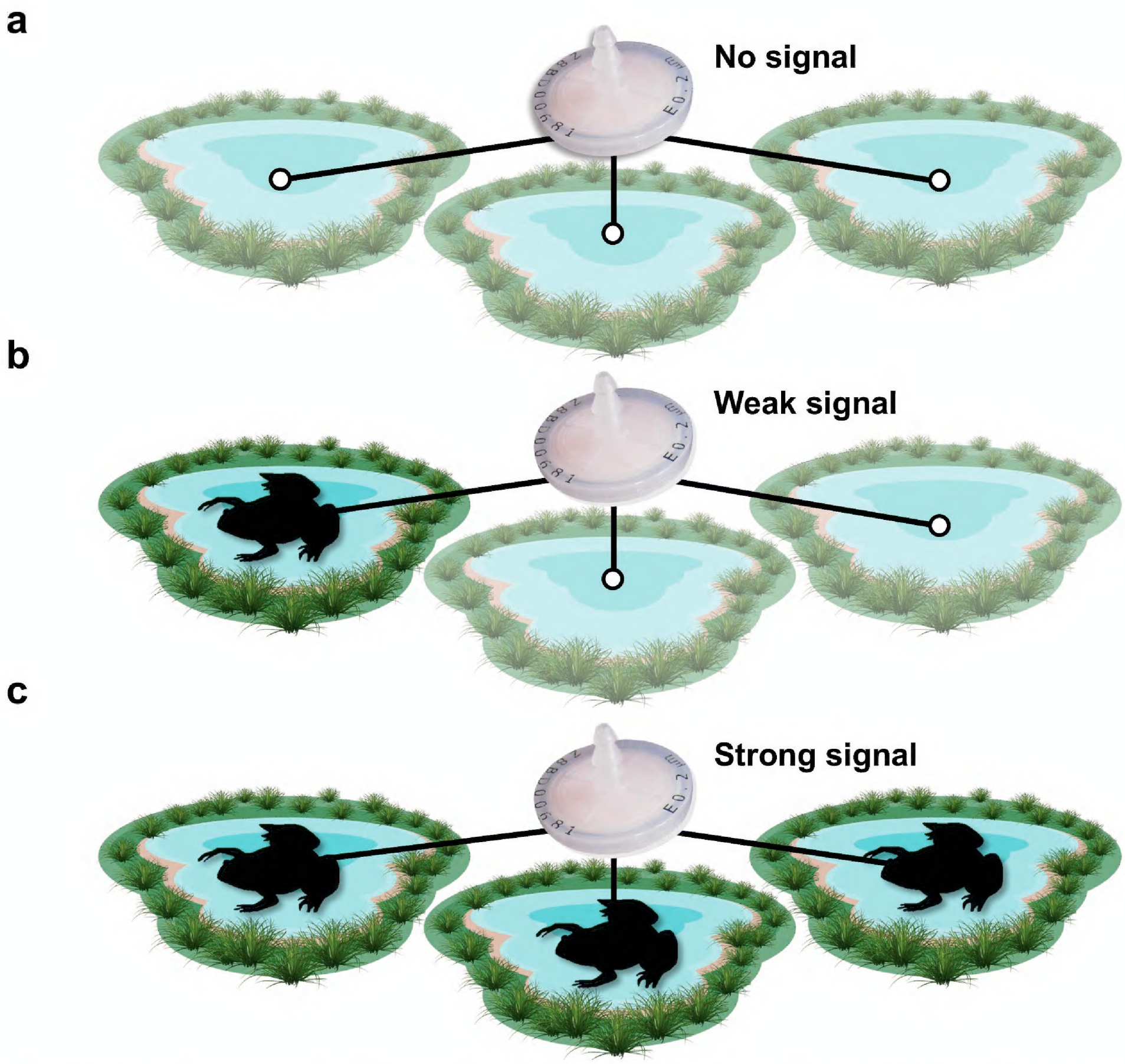


Figure 1. Schematic overview of pooling eDNA samples across locations under three different scenarios. In this hypothetical example, three ponds were sampled and filtered through a single filter. **a.** When *X. laevis* is absent in all three ponds, no *X. laevis* eDNA is detected (i.e, no signal). This could also occur when *X. laevis* is present in very low densities (i.e., false-negative); **b.** When *X. laevis* is present in a small proportion of the sampled ponds, a low amount of *X. laevis* eDNA is expected to be amplified (i.e., weak signal), unless *X. laevis* density and/or the ratio target-positive to target-negative ponds are high; **c.** When *X. laevis* is present in all three sampled ponds, in either low or high densities, a large amount of eDNA is expected (i.e., strong signal).

ous ponds, typically used for cattle or irrigation, which are known to be suitable habitats for *X. laevis* (Lobos and Jaksic 2005; Faraone et al. 2008; Rebelo et al. 2010). Given the aquatic nature of *X. laevis*, the river Lys and the Douvebeek are assumed to serve as dispersal corridors between France and Belgium (Adriaens et al. 2023; Pauwels et al. 2023).

The Lys is a partially canalized river, extending 202 kilometres in total length. Within the study area, the river ranges from 30 to 70 metres in width and reaches a maximum depth of 4.5 metres (Fig. 2). It originates in Pas-de-Calais, France, and flows northeast along the French-Belgian border before draining in the river Scheldt in Belgium. The Douvebeek is a small tributary of the river Lys that is

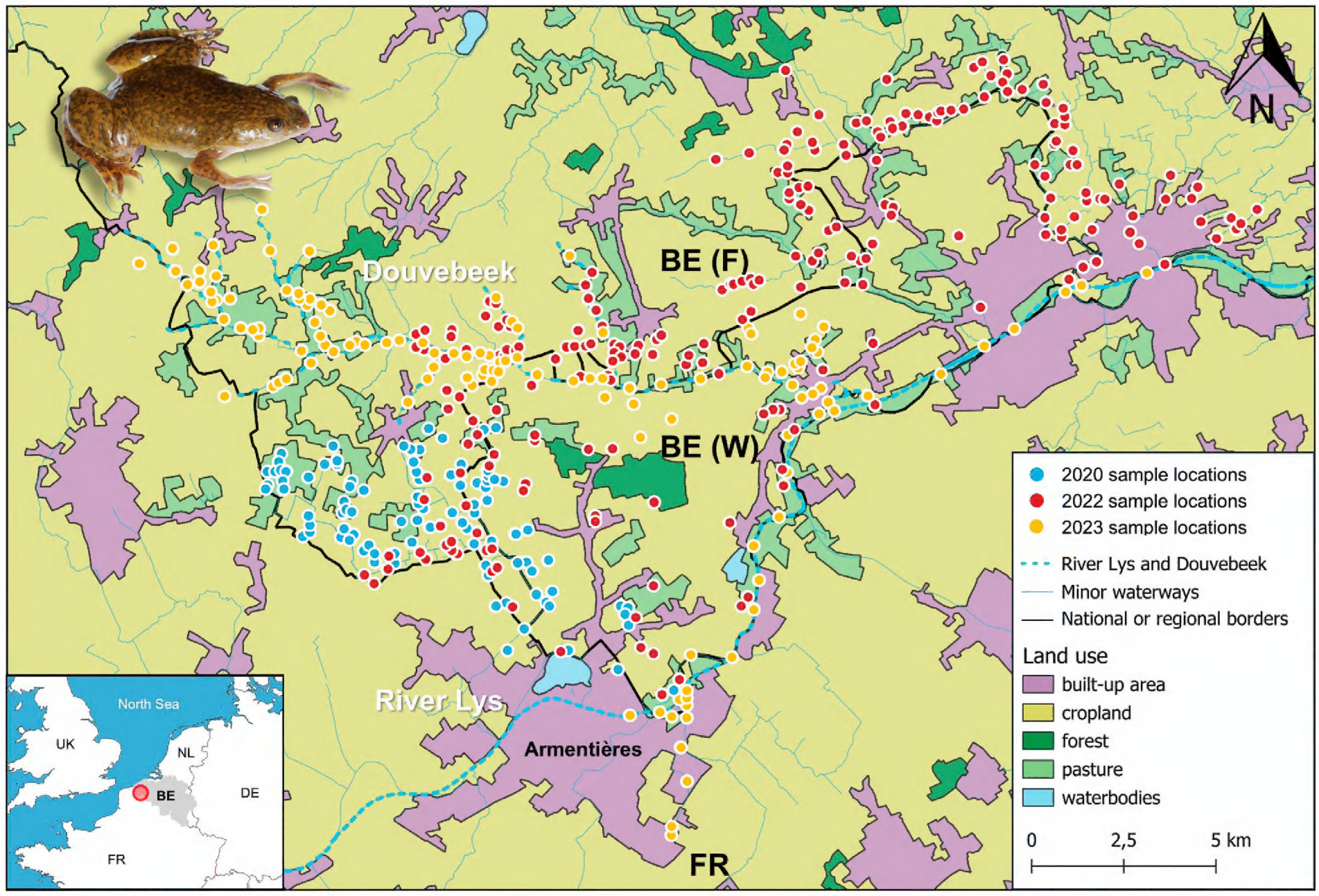


Figure 2. Map of the sampled area along the French-Belgian border. France is labelled as "FR", while the two Belgian Regions, Wallonia and Flanders, are labelled "BE(W)" and "BE(F)", respectively. Coloured circles indicate the sampled locations per sampled year. The river Lys and the Douvebeek are depicted as thick blue lines, with other waterways represented by thinner blue lines.

approximately 21 kilometres long, originating along the hillside of the Mont Noir in France. This stream continues southeast into Flanders, where it flows along the Flemish-Walloon border, draining into the river Lys in the Walloon municipality of Comines-Warneton. The Douvebeek can be characterized as a turbid creek with discharge levels that strongly fluctuate in response to precipitation events. The source area is considered ecologically valuable with several adjacent natural areas harbouring high biodiversity including large pondscapes with important relict populations of native amphibians, such as four local newt species, including the protected great crested newt *Triturus cristatus* (Laurenti, 1768), which are vulnerable to *X. laevis* invasion (Lillo et al. 2011; Courant et al. 2018). The middle and lower reaches of the stream, however, have poor water quality due to erosion, wastewater discharge, and nutrient inflows.

Sampling

Both lentic (i.e., ponds) and lotic (i.e., ditches, streams, canals, and rivers) systems were sampled for eDNA in April 2020 (86 sites), August 2022 (211 sites), and August 2023 (129 sites). These sampling periods coincide with the reproductive season and peak activity of *X. laevis*, which spans from March to October, thereby increasing the likelihood of detection (Courant et al. 2018; Everts et al. 2021). Sampling locations covered the French-Belgian border region (i.e., in Flanders, Wallonia, and France), mainly focusing on the river Lys, the Douvebeek, and the

surrounding areas (Fig. 2). In 2020, sampling focused on the western part of the region, based on the assumption of overland cross-border dispersal. Results from this initial campaign informed the 2022 sampling strategy, which shifted to the eastern part of the study area and considered both overland and lotic dispersal pathways. In 2023, the sampling design was further refined based on insights from previous years, with a focus on evaluating the role of the Douvebeek and river Lys as potential corridors for transboundary dispersal (Fig. 2).

In total, 303 lentic and 123 lotic sites were sampled. To minimize the number of required eDNA analyses while maintaining spatial coverage, we implemented a novel sampling strategy. This strategy involved pooling water from multiple sampling locations (mean: 4.4, range: 2–8), and filtering this pooled water through a single eDNA filter (i.e., pooling across locations). Adjacent ponds (mean distance to centroid: 309 m, range 7–1,333 m), as well as segments and tributaries of the Douvebeek and river Lys (mean distance to centroid: 632 m, range 52–2,692 m), were therefore grouped into sampling clusters. Within each cluster, water samples were merged into one integrated water sample (Fig. 1). This sampling strategy was used in areas with uncertain prior knowledge of *X. laevis* presence, and allowed us to assess its occurrence in 366 sites using only 83 eDNA filters. An additional 59 sites, located in regions where *X. laevis* presence was considered more likely (e.g., the downstream portion of the Douvebeek, proximal to waterways) were individually sampled, yielding a grand total of 142 eDNA filters. To evaluate the performance of the pooled sampling approach, we selected two clusters, each comprising four ponds, of which the initial pooled sample indicated the presence of *X. laevis* DNA. These ponds were then sampled both individually and again via pooled sampling, generating an additional ten eDNA filters for analysis.

Upper water layers were sampled for eDNA from the shore, using a sterile sampling pole with a 0.5 L sterile bag attached at the end. As eDNA particles are heterogeneously distributed in lentic systems, detection sensitivity was increased by implementing a merged sampling (i.e., pooling of water within a location), as described by Brys et al. (2021). As such, water was collected from all sites attributed to a single sampling cluster, and immediately filtered in the field over a single disc filter containing a 0.8 µM PES membrane combined with a 5 µm glass fibre prefilter (NatureMetrics, Surrey, England). Water was filtered until clogging, and the total volume of filtered water was recorded. Filters were then sealed off at both ends and immediately placed in a freezer box. At the end of the day, the filters were transferred to storage at -21 °C, where they remained until molecular analyses were conducted. All reusable sampling material (i.e., sampling pole and bucket) was disinfected with a 2% Virkon S solution (Antec DuPont, Suffolk, UK) to minimize the risk of DNA cross-contamination. To test for potential contamination during field sampling, five negative controls were included by filtering 2 L of mineral water in the field (i.e., field blanks): one at the end of the 2020 sampling campaign and one at the end of each of the two sampling rounds in both the 2022 and 2023 sampling campaigns.

Molecular analyses

All eDNA samples were stored and processed in a PCR-free building dedicated to low copy number template extractions. This facility hosts DNA-free high-efficiency particulate air (HEPA)-filtered compartments with positive pressure to prevent eDNA sample contamination. To test for PCR inhibition and to evaluate the ex-

traction efficiency of each filter, an internal positive control (IPC) was added in the first step of the extraction together with the lysis buffer (for details on the IPC see Everts et al. 2021). DNA extraction was performed using Qiagen's DNeasy Blood & Tissue Kit and purified with the DNeasy PowerClean Cleanup Kit (Qiagen) according to the guidelines provided by the manufacturer. To determine *X. laevis* presence and estimate its abundance, an 83-bp barcode located on the 12S region of the obtained DNA extracts was analysed and quantified via droplet digital PCR (ddPCR) using the primer/probe assay developed by Secondi et al. (2016). Even though this assay cannot discriminate between 12 (sub)species of *Xenopus*, it is expected that only *X. laevis* currently occurs in the sampled area (Pauwels et al. 2023), and thus a positive detection indicates the presence of *X. laevis*. For each eDNA sample, an average of 3 (range: 1–6) replicate ddPCR reactions were conducted. The unequal number of replicates across samples reflects the adaptive nature of the EDRR framework in which this study was conducted, where resource allocation was adjusted in subsequent years based on field conditions and preliminary findings. Specifically, a higher number of replicates was generally analysed for samples collected in areas with limited prior knowledge of species presence (e.g., locations increasingly distant from the known French population in Armentières), in more inland sites given the expectation that *X. laevis* primarily disperses via aquatic systems, and in larger water bodies where detecting a few individuals would require increased analytical effort (e.g., the river Lys compared to small ponds). To test for potential PCR failure or contamination, each ddPCR plate included two positive controls containing 10 pg/µL of DNA extracted from *X. laevis* tissue, along with no-template controls (NTCs) that served as PCR blanks by omitting DNA from the reaction. The advantages of ddPCR compared to conventional quantitative PCR (qPCR) are fourfold: (i) it eliminates the need for calibration curves through absolute quantification, (ii) its high level of sample partitioning enables highly precise measurements, (iii) it reliably detects low concentrations of target DNA even in the presence of abundant non-target DNA, and (iv) it exhibits greater resilience to PCR inhibitors, making it particularly suitable for complex environmental samples (Guri et al. 2024).

Data analyses

Xenopus laevis was considered present if successful PCR amplification occurred in at least one ddPCR replicate. Target eDNA concentrations (in copies/µL per L filtered water), used as a proxy for species density, were calculated following Everts et al. (2022), and averaged across replicates. The results of pooled samples (i.e., *X. laevis* presence/absence and target eDNA concentration) were plotted as the centroid of all the sites attributed to a single sampling cluster, representing a conservative approach to visualizing pooled eDNA data. We tested the effectiveness of pooled eDNA sampling for two clusters each containing four ponds. Specifically, we compared (i) the presence/absence of *X. laevis* in each individually sampled water body with its detection in the pooled sample, and (ii) whether the average target eDNA concentrations from individually sampled ponds corresponded to the target eDNA concentration in the pooled sample, functioning as measures of similarity across sampling methods.

To quantify the spatial distribution of *X. laevis* in the sampled area, we calculated a buffer-based approximation of the potentially occupied range. This

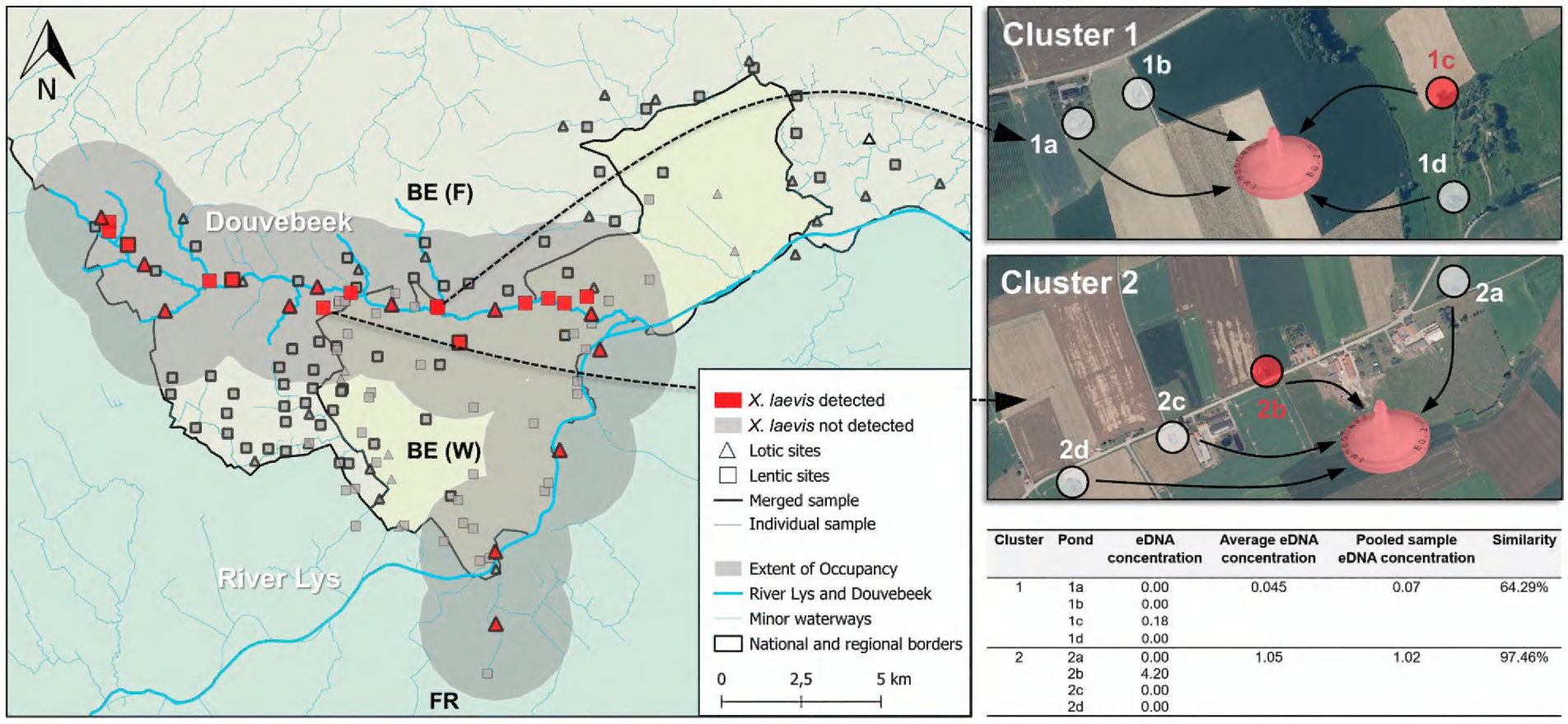
approach incorporates the locations of positive detections and the known dispersal capacity of the species to delineate areas where *X. laevis* may plausibly be present. Although the dispersal capacity of *X. laevis* varies widely (Pagano et al. 2024), we adopted a reasonable estimate of 2 km per year. This is considerably lower than the maximum predicted dispersal speed of 5.4 km in Chile, yet slightly higher than the 1.2 km per year reported in Southern France (Pagano et al. 2024), ensuring that the potential invaded area is not underestimated. Buffers with a 2 km radius were constructed around each target-positive eDNA detection (i.e., either the centroid for pooled samples or the exact locations for individual samples). Overlapping buffers were merged and the total surface area was calculated. The number of resulting polygons can be informative on the historical trajectory of the invasion, including the occurrence and number of introductions (Everts et al. 2023).

In order to facilitate the visualization of the merged eDNA sampling, we generated heatmaps using the kernel density estimation tool in QGIS v3.34.7. The obtained ddPCR data from each of the ponds and streams were analysed collectively to obtain an overall understanding of the invasion in the studied area. Given the highly localized nature of eDNA signals in small streams (Van Driessche et al. 2022), we expect the results from lotic systems to primarily reflect local conditions, rather than an accumulation of upstream signals (cf. Deiner et al. 2016). Weight was given to either the presence (0 when *X. laevis* was not detected or 1 when *X. laevis* was detected) or the log-transformed target eDNA concentrations in two separate heatmaps. Both heatmaps were generated using a 2 km linear decay, meaning that the influence of any given point on the heatmap value diminishes linearly to zero at a distance of 2 km. Other settings were left at default. Whereas this approach does not account for uneven sampling density, weighting by species presence or target eDNA concentration allows the generated heatmaps to provide an approximate qualitative (interpolation of *X. laevis* presence) and quantitative (i.e., interpolation of *X. laevis* eDNA concentrations) overview of the invaded area. However, in cases where sampling sites are excessively spatially clustered, we recommend employing more advanced interpolation methods that account for the uneven density of sampling locations, such as kriging or spatially explicit generalized linear mixed models (GLMMs).

Results

The generated ddPCR data exhibited high quality, with no DNA detected in field or PCR blanks, consistent recovery of expected target DNA concentrations from positive controls, and successful IPC amplification confirming the absence of PCR inhibition. Consequently, the likelihood of false positive detections due to laboratory or field contamination, as well as false negative detections resulting from extraction and PCR failure was considered unlikely.

Individual sampling of each of the four ponds within the two pond clusters that were selected for the pooled sampling validation and tested positive on *X. laevis* presence, revealed that *X. laevis* was present in only one pond per cluster (Fig. 3). Despite a substantially lower target eDNA concentration of that pond in one cluster compared to the other (0.18 vs 4.20 copies/µL per L filtered water), *X. laevis* eDNA could clearly be detected in the pooled sample of both clusters. Tar-



Cluster	Pond	eDNA concentration	Average eDNA concentration	Pooled sample eDNA concentration	Similarity
1	1a	0.00	0.045	0.07	64.29%
	1b	0.00			
	1c	0.18			
	1d	0.00			
2	2a	0.00	1.05	1.02	97.46%
	2b	4.20			
	2c	0.00			
	2d	0.00			

Figure 3. The *X. laevis* invasion along the French-Belgian border. Samples of streams and ponds are represented by triangles and squares, respectively. For pooled samples, the centroid of the sampled locations is mapped. *Xenopus laevis* detection is indicated in red, while its non-detection is shown in grey. The grey shading represents the buffer-based occupied area and was constructed with a buffer of 2 km around the target-positive locations. Insets display two clusters where the pooled sampling methodology was empirically tested. White and red dots represent ponds where the individual sample was negative and positive, respectively. The red-coloured filter represents a target-positive pooled sample. The table shows *X. laevis* eDNA concentrations (expressed in copies/μL), the average concentration across individually sampled ponds, the concentration of the pooled sample, and the similarity between the latter two.

get eDNA concentration of the pooled sample in the higher-concentration cluster (1.02 copies/μL) closely matched the average concentration of the individual samples (1.05 copies/μL; 97.5% similarity). This similarity was less pronounced in the lower-concentration cluster (pooled sample: 0.07 copies/μL; average of individual samples: 0.045 copies/μL; 64.3% similarity).

Xenopus laevis DNA was detected in 26 of the 142 collected samples (12 lotic and 14 lentic; 11 individual and 15 pooled samples; 0 in 2020, 3 in 2022, and 23 in 2023), resulting in a total occupied area of 103 km^2 within the study area (Fig. 3; Suppl. material 1). The spatial proximity of positive *X. laevis* detections resulted in a single, continuous polygon, suggesting the presence of one interconnected population. All positive detections occurred within one kilometre of either the river Lys or the Douvebeek. The distribution of *X. laevis* extends from the headwaters of the Douvebeek to its confluence with the river Lys, and upstream along the river Lys and a small tributary (Courant du Pont Bertin) near Armentières, encompassing both France and the Walloon and Flemish Regions of Belgium. The frequency of positive detections and *X. laevis* eDNA concentrations were higher in the Douvebeek than in the river Lys (Fig. 4). Positive detections were relatively evenly distributed within the Douvebeek, whereas the majority of samples exhibiting high target eDNA concentrations were located more downstream near its confluence with the river Lys. Nine sites (6 lentic, 3 lotic; 3 sampled in 2022, 6 sampled in 2023) exhibited target eDNA concentrations similar to or exceeding those at the three known breeding sites reported in Adriaens et al. (2023); eight were near the Lys-Douvebeek confluence, and one lentic site was near the source of the Douvebeek (Suppl. material 1).

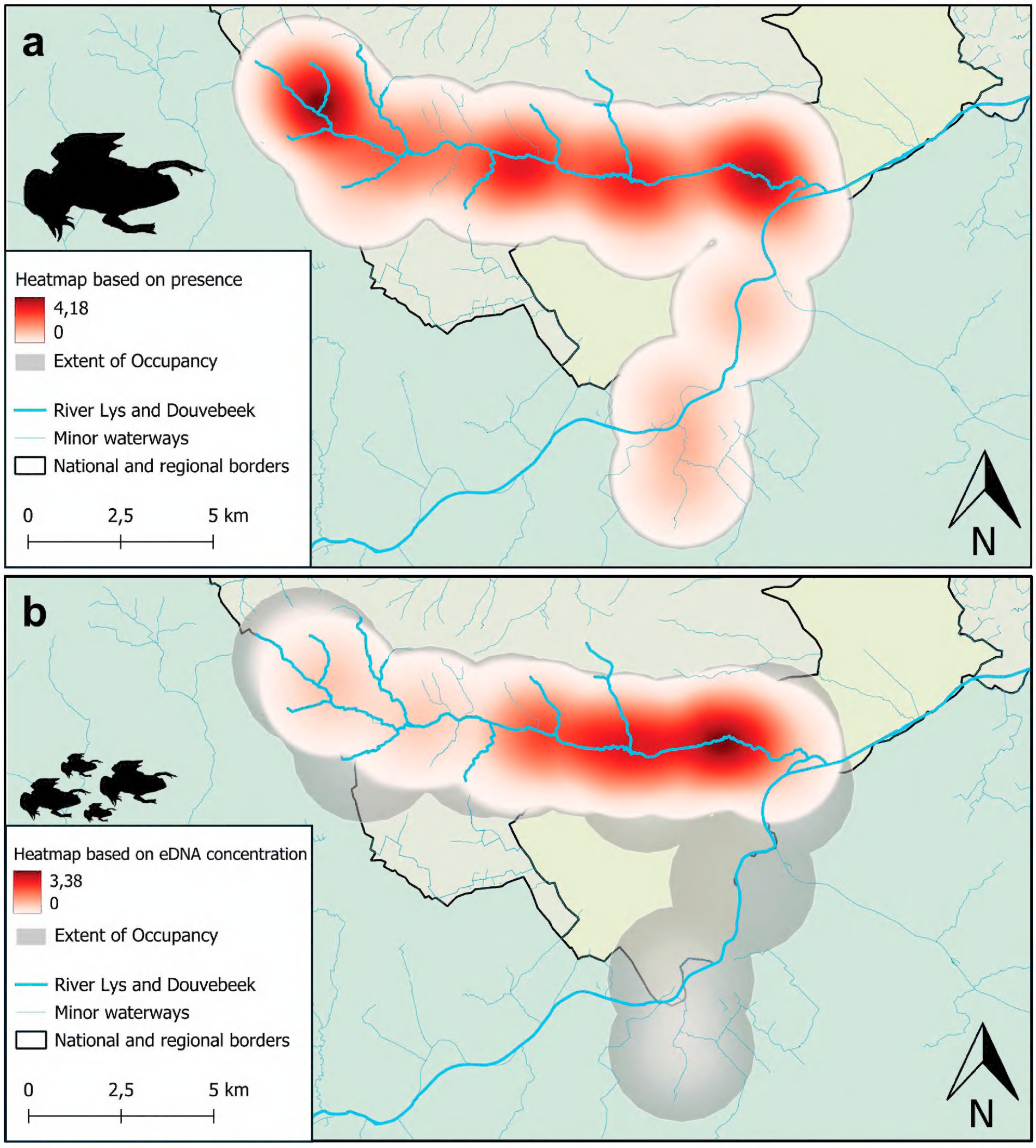


Figure 4. Heatmaps showing the *X. laevis* invasion in the French-Belgian border region based on (**a**) *X. laevis* detection determined using eDNA, and (**b**) density as approximated by target eDNA concentrations.

Discussion

The globally increasing number of newly arriving non-native invasive species (NIS) calls for efficient and large-scale early-detection-rapid-response (EDRR) systems to alert for incipient invasions and increase the likelihood of successful eradication (Hulme 2006; Vander Zanden and Olden 2008; Seebens et al. 2017). Pivotal to their effectiveness are monitoring techniques that maximize search coverage and minimize costs (Reaser et al. 2020). While eDNA-based techniques have

significantly advanced aquatic species monitoring (Everts et al. 2023; Greenhalgh et al. 2022), related expenses can inflate substantially when information for spatial prioritization – such as invasion pathways, spread patterns, and habitat preferences – is limited (Epanchin-Niell et al. 2014; Trebitz et al. 2017; Morisette et al. 2020). To address this, we developed a novel eDNA-based sampling strategy that increases spatial coverage while reducing analytical costs and maintaining adequate detection probability to support rapid response. We demonstrate the preliminary effectiveness of this sampling strategy using the emerging *X. laevis* invasion in the French-Belgian border region as a case study.

Pooled eDNA sampling

Effective early-detection-rapid-response systems require substantial sampling efforts (Reaser et al. 2020). A large proportion of collected samples may yield negative detections, either because the species has not yet reached the targeted area or due to spatiotemporal mismatches in sampling and species presence (Epanchin-Niell et al. 2014; Morisette et al. 2020). As the primary aim of most EDRR systems is NIS detection across broad geographical areas – transcending national or administrative borders – rather than focusing on specific locations such as particular ponds or sections of streams or ditches, workload and costs can be drastically reduced by pooling water samples from multiple locations for eDNA-based analyses (Fig. 1). A similar approach is already employed in (i) wildlife pathogen monitoring, where samples from multiple individuals within the same population are pooled for DNA extraction (Hyatt et al. 2007), (ii) population genetics, where DNA from multiple individuals is pooled for sequencing (Anand et al. 2016), or (iii) eDNA sampling of large water bodies, where subsamples from various sites within a given water body are pooled for analysis (i.e., pooling within a location) (Brys et al. 2021). Empirical testing demonstrated that *X. laevis* remained detectable in pooled samples (i.e., pooling across locations) encompassing four ponds, even when present at low densities in a single pond. Furthermore, the target eDNA concentrations in these pooled samples approximated the average concentrations from the individually sampled water bodies. However, variability increased in the lower-concentration cluster, potentially due to increased stochasticity commonly associated with lower target eDNA concentrations (Brys et al. 2023). Although further validation is required to confirm its reliability and broader applicability, pooled eDNA samples and corresponding eDNA concentrations of a target species likely provide useful proxies for assessing the overall presence and average NIS density across various sampled locations (Yates et al. 2019; Carvalho et al. 2022; Everts et al. 2022).

Pooling eDNA samples from multiple locations can significantly reduce the total number of samples required, accelerating both sample collection and molecular processing, leading to substantial time and cost savings. However, recognizing the limitations of this sampling technique is key to its appropriate deployment. For instance, a positive detection in a pooled sample provides no information about how many and which exact locations are invaded – the target species could be present in all, a subset, or only one of the sampled locations (Hyatt et al. 2007). Another constraint is that when the targeted NIS is present in a small proportion of the locations within a pooled sample or when clean water devoid of PCR inhibitors is limited to a subset of those locations, dilution effects and PCR inhibition, respectively, can result in false-negative detections (i.e., falsely concluding the absence

of a species), particularly when species density is low (Sato et al. 2017; Zhang et al. 2020). To mitigate this risk, filtering larger volumes of water can increase the quantity of DNA captured (Capo et al. 2020), multiplexing multiple markers targeting the same species can enhance detection sensitivity without substantial additional cost (Brys et al. 2023), and using a ddPCR platform combined with a DNA purification step during analytical processing can largely mitigate the impact of PCR-inhibiting compounds (Everts et al. 2021; Guri et al. 2024). Importantly, the likelihood of false-negative detections increases with the proportion of target-negative locations included in a pooled eDNA sample, which, in turn, may be influenced by various factors, including the biology of the target species (e.g., phenology), habitat suitability, and the spatial positioning of sites within the invaded range (i.e., core vs edge). Hence, the optimal number of pooled locations is context dependent and must be carefully assessed. Notwithstanding these limitations, we argue that pooled eDNA sampling is particularly valuable for mapping emerging invasions across large geographic areas, where the trade-off between geographical coverage and detection sensitivity tilts towards the former. Once the presence of the target species at a given location (cluster) is confirmed, sampling efforts can transition to single-site eDNA sampling to refine the understanding of fine-scale distribution patterns and to better inform management interventions.

Beyond the early invasion stage

Based on climatic modelling approaches, the French-Belgian border region has previously been considered as climatically unsuitable for the long-term establishment of *X. laevis* (Measey et al. 2012; Ihlow et al. 2016; Scalera et al. 2019). Indeed, unfavourable climatic conditions have been associated with the extinction of established *X. laevis* populations in southern parts of the United Kingdom, which share a similar climate to northern France and Belgium (Tinsley et al. 2015). However, our findings reveal that *X. laevis* is firmly established and more widespread in the French-Belgian border region than previously anticipated (Pauwels et al. 2023), suggesting that the species can thrive even under predicted suboptimal climatic conditions. More specifically, we detected *X. laevis* in 26 out of 142 samples, estimating its currently occupied area to be 103 km^2. The invasion remains restricted to lotic systems and adjacent lentic habitats, with limited evidence of colonization in more distant lentic environments. Indeed, the probability of pond colonization by *X. laevis* typically significantly decreases with distance from lotic systems (Measey et al. 2012). The current invaded range of the studied case covers an area similar in size to the Portuguese population that was introduced in the 1980s (Rebelo et al. 2010; Moreira et al. 2017), but is substantially smaller than the population in western France, which has expanded to 8,400 km^2 since 1981 (Pagano et al. 2024).

The invasion history in the French-Belgian border region is undocumented. The extensive occupied area suggests that the invasion has progressed beyond an early stage, implying that the initial introduction likely occurred years, if not decades ago. Indeed, due to the cryptic nature of *X. laevis*, substantial delays between introduction and first detection are common, with some cases extending up to 25 years (Measey et al. 2012). The earliest documented record of *X. laevis* in Belgium dates back to 2006 (though this record was not publicly available until 2019) and was located just north of Armentières (France), suggesting that the initial introduction occurred at least 19 years ago. The spatially continuous occupied area, coupled

with the low probability of pet releases in sparsely populated locations such as agricultural areas (Banha et al. 2017), suggests that the *X. laevis* invasion at the French-Belgian border originated from a single introduction and now constitutes one interconnected population (Everts et al. 2023; Pauwels et al. 2023). Due to the lack of operational systematic, transboundary monitoring systems for detecting incipient invasions (Pergl et al. 2020; Haubrock et al. 2025), we consider the window of opportunity (*sensu* Booy et al. 2020) to eradicate *X. laevis* entirely from this region to have narrowed considerably.

Measured target eDNA concentrations varied significantly across both lotic and lentic systems. A considerable number of samples exhibited high target eDNA concentrations, indicating large species densities and most likely reproductive activity (Everts et al. 2022). Ponds exhibiting high target eDNA concentrations were mainly those used for agricultural purposes. In addition to seasonal desiccation, the periodic draining of these ponds for irrigation purposes may prompt *X. laevis* to migrate to other water bodies, potentially accelerating the pace of invasion (Lobos and Jaksic 2005). Furthermore, the agricultural landscape surrounding many of these ponds promotes high nutrient levels, creating conditions that favour *X. laevis* proliferation (Tinsley and Kobel 1996). While the highest overall target eDNA concentrations were detected in the downstream area of the Douvebeek, target eDNA concentrations potentially indicative of breeding (i.e., similar in magnitude to the known breeding sites reported in Adriaens et al. 2023) were also found in ponds near the stream's source. Although *X. laevis* reproduction in the valley of the Douvebeek was previously documented (Adriaens et al. 2023; Pauwels et al. 2023), indications of reproduction near its source are particularly alarming due to its proximity to the Yser catchment and the risk of further expansion into ecologically valuable habitats.

Lotic systems serving as dispersal corridors

Our results provide strong evidence that the river Lys and the Douvebeek serve as dispersal corridors for *X. laevis* in the French-Belgian border region (Pauwels et al. 2023). Amphibian invasions indeed frequently follow the course of river valleys (Everts et al. 2025), largely due to the increased prevalence of lentic habitats, such as ponds and ditches, and the lower energetic cost of movement in aquatic versus terrestrial environments (Vimercati et al. 2024). While overland movement, particularly through croplands, contributes to *X. laevis* dispersal, its spread is significantly accelerated by the presence of aquatic corridors such as irrigation channels and small creeks (Fouquet and Measey 2006; Measey 2016; Vimercati et al. 2019). Notably, *X. laevis* eDNA was not detected in samples from the river Lys downstream of its confluence with the Douvebeek, suggesting that migrating *X. laevis* individuals cross the French-Belgian border via the upstream part of the river Lys and the Douvebeek (Pauwels et al. 2023). While our results do not allow us to infer the directionality of the invasion, it is likely that *X. laevis* invaded the Douvebeek in an upstream direction, suggesting that its spread is not limited to downstream movement.

The number of positive detections and target eDNA concentrations are substantially higher in the Douvebeek compared to the river Lys. This difference possibly reflects the distinct biotic and abiotic conditions of both systems, but may also be influenced by variations in hydrological transport processes that influence target eDNA concentrations (Van Driessche et al. 2022). The canalized river Lys, being

larger in size and having higher discharge rates, is characterized by steep banks that may hinder *X. laevis* exiting the water. Additionally, the surrounding built-up environment offers limited availability of suitable breeding habitats in the area (Lobos et al. 2013; Hill et al. 2017). Conversely, the Douvebeek and its tributaries are characterized by vegetated, natural banks and a network of adjacent artificial, turbid, and eutrophic ponds, and therefore may provide a more favourable environment for sustaining a large, viable population of *X. laevis* (Lobos et al. 2013). These conditions, similar to those in other regions with rapidly expanding *X. laevis* populations (Measey et al. 2012), may have facilitated the establishment and spread of *X. laevis* in the French-Belgian border region, despite suboptimal climatic conditions (Measey et al. 2012; Ihlow et al. 2016).

Towards effective, eDNA-informed, transboundary management

Since August 2024, *X. laevis* has been listed as a NIS of European Union Concern, necessitating coordinated action at the Union level under EU Regulation 1143/2014. Despite the regulation's clear potential to deliver continental-level conservation gains (Genovesi et al. 2015; Tollington et al. 2017), the French-Belgian *X. laevis* invasion exemplifies several persistent obstacles to transboundary action (Magliozzi et al. 2024).

Effective management warrants actions to be undertaken across the entire affected area, yet a major challenge lies in engaging all affected stakeholders and decision-makers across administrative boundaries (Adriaens et al. 2015). To date, despite a management recommendation from the scientific community (Reniers et al. 2023), no explicit management decisions have been implemented in any of the affected regions. The rapid transboundary assessment of the invaded area facilitated by the cost-effective eDNA sampling method presented in this study will hopefully advance this process by providing a timely oversight of the invaded area, emphasizing the urgency for transboundary action, and offering a stronger foundation for policy discussions and coordinated intervention.

Fragmented, small-scale, and uncoordinated management interventions must be replaced by systematic, harmonized, cross-border monitoring and management, underpinned by a centralized coordination and clearly defined responsibilities (Adriaens et al. 2015). Given the uncertainties surrounding management effectiveness, we recommend an adaptive, evidence-based course informed by integrated modelling approaches that incorporate spatiotemporal invasion dynamics, efficacy of various measures, and landscape configuration (cf. Ginal et al. 2021). In this context, seamless data exchange between jurisdictions and stakeholders, along with fast and transparent data visualization are essential to outlining and executing these varied responses (Groom et al. 2017; Cartuyvels et al. 2024). We therefore recommend implementing one formally adopted, standardized surveillance approach and sampling design to ease the interpretation of eDNA signals and harmonize monitoring across borders and habitats.

Improving monitoring capacity and data sharing is crucial but challenged by shortage of scientific capacity and inconsistent procedural approaches between countries and regions. Our study showed that *X. laevis* occupies both lentic and lotic elements of the transboundary hydrographic system, with the former likely serving as breeding sites and the latter as dispersal corridors (Moreira et al. 2017). These landscape elements are varied in nature, spanning natural as well as anthro-

pogenically disturbed habitats. Furthermore, considering its capacity for overland dispersal, there appear to be few implicit dispersal barriers for the species, and any wet habitat could potentially serve as a stepping stone (Vimercati et al. 2019; Ginal et al. 2021). Consequently, management approaches tailored to each of these varying conditions are needed (Adriaens et al. 2023; Reniers et al. 2023). The eDNA data gathered in this study can support such context-specific approaches in a cost-efficient and standardized way. For instance, ponds suspected of harbouring *X. laevis* populations could be targeted for eradication using quicklime treatments if the importance of complete removal exceeds that of possible collateral damage (Adriaens et al. 2023). Additional measures could include barrier-based dispersal control, or active trapping campaigns using fyke nets or bucket traps. Furthermore, eDNA data can inform the design of formal surveillance schemes and post-intervention monitoring. Treated areas can be screened for residual or re-invading individuals, while ponds can be strategically selected for trapping efforts, and areas within dispersal distance of known populations can be targeted for intensified surveillance (Everts et al. 2022, 2023).

Conclusion

The invasion of *X. laevis* along the French-Belgian border represents a considerable transboundary management challenge. Tackling this invasion necessitates coordinated action and decision-making across national and regional governments, requiring stakeholder involvement and the timely mobilization of dedicated resources (Magliozzi et al. 2024). eDNA-based analyses can be used to inform several critical steps of the adaptive management cycle, including determining invasion extent at the landscape level, quantifying ecological impacts, identifying management strategies, and evaluating management effectiveness (Everts et al. 2022, 2023, 2024). Pooled eDNA sampling offers the potential to promptly deliver information with sufficient resolution to inform decision making on management and can be invaluable to comparable aquatic invasion scenarios.

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Conflict of interest

The authors have declared that no competing interests exist.

Ethical statement

No ethical statement was reported.

Use of AI

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Author contributions

Conceptualization: RB, TE. Data curation: KK, TE. Formal analysis: KK, TE. Funding acquisition: TE, LD, RB, TA. Investigation: LD, TE. Methodology: TE, RB, SN. Project administration: RB, TA. Supervision: RB. Validation: SN, RB, KK, LD, TE. Visualization: TE, KK. Writing – original draft: TA, TE, LD. Writing – review and editing: SN, RB, JS.

Author ORCIDs

Teun Everts https://orcid.org/0000-0001-7862-4209
Loïc van Doorn https://orcid.org/0000-0003-3497-9168
Tim Adriaens https://orcid.org/0000-0001-7268-4200
Sabrina Neyrinck https://orcid.org/0000-0002-7379-9269
Jeroen Speybroeck https://orcid.org/0000-0002-7241-7804
Rein Brys https://orcid.org/0000-0002-0688-3268

Data availability

All of the data that support the findings of this study are available in the main text or Supplementary Information.

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Supplementary material 1

Summary of the digital droplet PCR results and the associated metadata

Authors: Teun Everts, Loïc van Doorn, Tim Adriaens, Sabrina Neyrinck, Kevin Kerckhofs, Jeroen Speybroeck, Rein Brys

Data type: docx

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